Structure-activity relationships of rationally designed AMACR 1A inhibitors

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Keywords: α-Methylacyl-CoA racemase (AMACR; P504S); drug lipophilicity; enzyme inhibitors; rational drug design; Structure-activity relationships.

Abstract

α-Methylacyl-CoA racemase (AMACR; P504S) is a promising novel drug target for prostate and other cancers. Assaying enzyme activity is difficult due to the reversibility of the 'racemisation' reaction and the difficulties in the separating epimeric products; consequently few inhibitors have been described and no structure-activity relationship study has been performed. This paper describes the first structure-activity relationship study, in which a series of 23 known and potential rational AMACR inhibitors were evaluated. AMACR was potently inhibited (IC_{50} = 400 - 750 nM) by ibuprofenoyl-CoA and derivatives. Potency was positively correlated with inhibitor lipophilicity. AMACR was also inhibited by straight-chain and branched-chain acyl-CoA esters, with potency positively correlating with inhibitor lipophilicity. 2-Methyldecanoyl-CoAs were *ca.* 3-fold more potent inhibitors than decanoyl-CoA, demonstrating the importance of the 2-methyl group for effective inhibition. Elimination substrates and compounds with modified acyl-CoA cores were also investigated, and shown to be potent inhibitors. These results are the first to demonstrate structure-activity relationships of rational AMACR inhibitors and that potency can be predicted by acyl-CoA lipophilicity. The study also demonstrates the utility of the colorimetric assay for thorough inhibitor characterisation.

Abbreviations used: AMACR, α-methylacyl-CoA racemase; CDI, carbonyldiimidazole; DAST, (Diethylamino)sulfur trifluoride; DCC, Dicyclohexylcarbodiimide; DMAP, (dimethylamino)pyridine; MCR, 2-methylacyl-CoA racemase from *M. tuberculosis*; SAR, Structure-activity relationships; THF, tetrahydrofuran; TMSCl, chlorotrimethylsilane.

Introduction

Branched-chain fatty acids (*e.g.* phytanic acid pristanic acids) are common components of the human diet, and derivatives of such compounds are used as drug molecules *e.g.* Ibuprofen [1, 2]. Degradation of branched-chain fatty acids occurs as the acyl-CoA ester, and the acyl-CoA oxidases and other enzymes involved in β-oxidation have an absolute requirement for *S*-2-methylacyl-CoAs [3-5]. However, *R*-2-methylacyl-CoAs are produced from dietary and endogenous fatty acids and these cannot be immediately degraded by β-oxidation. The enzyme α-methylacyl-CoA racemase [1, 2] (AMACR; P504S; E.C. 5.1.99.4) catalyses conversion of *R*-2-methylacyl-CoAs to a near 1:1 epimeric mixture [6, 7] by a deprotonation / reprotonation reaction [7, 8], probably *via* an enolate intermediate [9] (this reaction is referred to as “racemization” [10]). The resulting *S*-2-methylacyl-CoAs are degraded by β-oxidation whilst the 2*R* epimers are further processed to the 2*S* epimers by AMACR [1, 2]. AMACR also plays a key role in the *in vivo* pharmacological activation of *R*-Ibuprofen to *S*-Ibuprofen, via the corresponding acyl-CoA esters [1, 2, 11]. The *S*-Ibuprofen resulting from this pathway exerts its anti-inflammatory effect by inhibiting cyclooxygenase-1 and -2 [12].

AMACR protein levels are increased in prostate [13, 14] and several other cancers [1, 15-18]. Catalytic activity of AMACR is increased by 4 to 10-fold in prostate cancer cells [19, 20], with the AMACR 1A splice variant [1, 2, 21-24] (possessing “racemase” activity [7, 10]) showing the most significant increase in expression [19, 20]. Reducing AMACR 1A levels using siRNA or shRNA approaches [19, 25, 26] has been shown to reduce proliferation of prostate cancer cells *via* a pathway which is synergistic with the use of an androgen

receptor antagonist, studies which have validated AMACR 1A as a chemotherapeutic target. Some advanced prostate cancer cell lines revert from castrate-resistant (a.k.a. androgen-independent) growth to androgen-dependent growth upon knockdown of AMACR 1A [26]. Consequently, AMACR has attracted considerable interest as a prostate cancer biomarker [1, 2, 27] and drug target [25, 28-31]. However, the lack of a convenient assay to measure AMACR activity [32, 33] has severely hampered the development of AMACR inhibitors as new chemotherapeutic drugs against cancers that over-express AMACR, and consequently only a few rationally designed inhibitors of AMACR [28-30, 34] or MCR [31, 35] (*M. tuberculosis* homologue) have been reported. No systematic study of AMACR inhibitor SAR has been undertaken [32, 33].

Recently, we reported a versatile continuous assay for AMACR based on the utilisation of our novel substrate **1** that can eliminate 2,4-dinitrophenolate **2**, which can be monitored by absorbance at 354 nm, and unsaturated product **3** (Scheme 1). This new assay [32] was used to examine the potency of two known acyl-CoA inhibitors (*N*-dodecyl-*N*-methylcarbamoyl-CoA **4** [29] and ibuprofenoyl-CoA **5** [6]; Figure 1) and selected known non-specific protein modification agents [25]. This paper reports the first systematic examination of SAR for rationally designed acyl-CoA inhibitors of AMACR. Compounds investigated (Figure 1) include those with aromatic side-chains, (**5** - **11**); Straight-chain acyl-CoA esters (**12** – **17**); Branched-chain substrates (**18** – **21**) and product **22**. Analogues of known inhibitors with modified 2-methylacyl-CoA moieties (**4**, **23** - **26**) were also examined. The results reveal a correlation between potency and lipophilicity of the inhibitors, consistent with observations on MCR inhibitors [35], the homologous enzyme from *M. tuberculosis*.

Results and discussion

AMACR is a promising novel cancer drug target, but therapeutic development in this field has been slow due to the lack of a robust enzyme assay. Thus, the majority of studies reporting AMACR inhibitors have largely focussed on rationally designed drugs [28-30, 32, 33]. In most cases, only one or a few examples of each inhibitor type has been evaluated, and no systematic SAR study has been performed. Initial SAR studies have been carried out on reversible [31] and irreversible [35] inhibitors of MCR (the *M. tuberculosis* homologue). In addition, different research groups have used different assays during their studies, making it difficult to compare results. In this study, the SAR of rational AMACR inhibitors were explored using a series of acyl-CoA esters (Figure 1). These included compounds previously tested as substrates (**6** – **11** [6, 11]; **12** – **17** [36]; **18** – **20**, **22** [7, 10]). Most of these compounds have not been tested as inhibitors with the exception of **5** [28, 32, 37, 38] and **13**, **15**-**17** (which were previously reported to be inactive [37, 38]). Compound **21** was included as an epimer of **20**, and has not been previously reported as a substrate or inhibitor (although the 3-fluoro-2-methyltetradecanoyl-CoA analogues are potent inhibitors [28]). Compound **24** is a synthetic intermediate to **25**, and has not been previously tested as a substrate or inhibitor. Compounds **22**, **25** and **26** are intermediates in the subsequent β-oxidation pathway [39], and have not been previously tested as substrates or inhibitors. Analogues of compounds **23**, and **25** with different side-chains have been previously tested as inhibitors of AMACR or MCR [9, 30]. Compound **4** was previously reported as the most potent AMACR inhibitor [29, 32], and is included as an acyl-CoA core analogue.

Chemical synthesis of acyl-CoA inhibitors

(2*S*,3*S*)-3-Fluoro-2-methyldecanoyl-CoA **21** was synthesised by an analogous route to (2*R*,3*R*)-3-fluoro-2-methyldecanoyl-CoA **20** [10, 28], using an Evans' auxiliary strategy (Scheme 2). Aldol-like reaction of deprotonated **27** with octanal gave the (2*S*,3*R*)-3-hydroxy-2-methyl intermediate **28**. From here, alcohol **28** was activated and replaced with fluoride with inversion of configuration, using DAST to give 3-fluoro-2-methyl derivative **29**. The reaction is thought to go with inversion of stereochemical configuration (by analogy with the work of Carnell *et al*. [28]). Removal of the Evans' auxiliary from intermediate **29** provided the carboxylic acid **30** under mild conditions that involved *in situ* generation of lithium hydroperoxide. Intermediate **30** was subsequently converted to the CoA thioester **21** using the standard synthetic method with *N*,*N*'-carbonyldiimidazole [6, 10, 11, 32, 33, 40]. This compound was stable in solution in the absence of AMACR, showing that the relative geometry of the α-proton and fluorine atom was *syn*- (*anti*- epimers rapidly eliminate fluoride, presumably by an E2 mechanism [10]).

2-Methylenedecanoyl-CoA **23** was synthesised by an adaptation of the method reported by Morgenroth *et al*. [30] (Scheme 3). Meldrum's acid **33** was acylated with octanoic acid **32** using DCC activation; the intermediate ketone was reduced to the octyl-Meldrum's acid **31** with sodium triacetoxyborohydride generated *in situ*. Subsequent reaction of **31** with Eschenmoser's salt gave the 2-methylene ester **34**. Base-hydrolysis furnished the 2-methylene acid **35**, which was coupled with CoA-SH by a mixed anhydride approach to give 2-methylenedecanoyl-CoA **23**.

2-Methyl-3-oxodecanoyl-CoA **25** was synthesised by the method of Reen *et al*. [41] from **36** (Scheme 4). The ketone in **37** was protected as the cyclic acetal

36. Hydrolysis of the ester group in **36** gave the corresponding acid **38**, which was then coupled with CoA [6, 10, 11, 32, 33, 40] to give **24**. Acidolysis of the acetal protection provided **25**.

2*S*,3*R*-3-Hydroxy-2-methyldecanoyl-CoA **26** was synthesised (Scheme 5) from the acyl-Evan's auxiliary **28** (Scheme 2, *vide supra*) by hydrolysis with lithium hydroperoxide to give acid **39**, which was converted to the CoA ester **26** using the standard procedure [6, 10, 11, 32, 33, 40].

Evaluation of inhibitors

The selected AMACR inhibitors were evaluated using the colorimetric assay [32]. Incubation of active human AMACR 1A with substrate **1** results in production of 2,4-dinitrophenolate **2** and unsaturated product **3** (Scheme 1). Hence, the potency of inhibitors can be determined based on measuring the absorbance of **2** at 354 nm. Inhibitory potency was assessed using dose-response curves to determine IC_{50} values (Figure 1).

As expected, ibuprofenoyl-CoA **5** and its derivatives **6** – **9** were inhibitors of the enzyme, with most having IC_{50} values of *ca*. 500 nM (Figure 1). Variation of the structure of the side-chain in these inhibitors appeared to make little difference to inhibitory activity (as judged by IC_{50} values), although fenoprofenoyl-CoA **6** appeared to be slightly more potent than the other examples and naproxenoyl-CoA **9** appeared to be slightly less so. All these compounds are known substrates of AMACR [6] and are predicted to behave as competitive inhibitors. Ibuprofenoyl-CoA **5** has been previously confirmed to be a competitive inhibitor of AMACR, with K_i = 60 nM [32], consistent with observations of other workers on the human and rat enzymes [28, 37, 38]. The mandelic acid

derivatives *R*- and *S*-2-hydroxy-2-phenylacetyl-CoA **10** and **11** were also modest inhibitors (Figure 1), binding approximately ten times less strongly than compounds **5** – **9**. Compounds **10** and **11** are not substrates of AMACR, since enzyme catalysed α-proton exchange does not occur [11]. This result with **10** and **11** also demonstrates that inhibitors can possess a 2-hydroxy- group in addition to the previously reported 2-trifluoromethyl- [28] and 2-chloro- [29] groups in place of the 2-methyl group. A wide range of aromatic inhibitor side-chains can therefore be accomodated by the enzyme, consistent with predictions made based on the MCR crystal structures [8] and biochemical data [6, 11].

Acyl-CoA esters **12** - **17**, possessing alkyl side-chains were also assessed as inhibitors (Figure 1). The potency of inhibition for acyl-CoA esters with side-chains of four carbons or fewer (**15** – **17**) is very weak, with low levels of inhibition (15-30%) observed even at very high inhibitor concentrations (100 μM). Inhibition increased as alkyl chain-length increased. This behaviour is consistent with that of straight-chain acyl-CoA esters acting as substrates [36], where increased levels of α-proton exchange are observed with increasing chain length. Our results showing inhibition of AMACR by straight-chain acyl-CoA esters contrasts with the early observations of Schmitz *et al.*, who reported that these compounds were not inhibitors of the native human and rat enzymes [37, 38].

Inclusion of a 2-methyl group on the inhibitor increased potency by about 3-fold (compounds **18** and **19** compared to **12**), again consistent with the finding that 2-methylacyl-CoA esters are much more efficient substrates than their straight-chain equivalents [36]. *R*-2-Methyldecanoyl-CoA **18** appeared to be a slightly more potent inhibitor than *S*-2-methyldecanoyl-CoA **19**, and this probably

reflects the physiological role of AMACR in the conversion of *R*-2-methylacyl-CoAs to their *S*-2-methylacyl-CoA epimers [1, 2].

2-Methyldecanoyl-CoA derivatives with more acidic α-protons are better inhibitors than their parent compounds (Figure 1). *R*,*R*-3-Fluoro-2-methyldecanoyl-CoA **20** was about 5-fold more potent than was *R*-2-methyldecanoyl-CoA **18**. A similar trend was observed with *S*,*S*-3-fluoro-2-methyldecanoyl-CoA **21** and *S*-2-methyldecanoyl-CoA **19**, although both of these compounds were slightly less potent than their *R*-epimers. The product of the reaction, *E*-2-methyldec-2-enoyl-CoA **22**, is also a potent inhibitor. It is therefore difficult to determine if the observed IC_{50} values for **20** or **21** reflect the conversion of these substrates [10], product inhibition by **22** or both. These observations contrast with early studies [37, 38], which suggest that **22** was not an inhibitor of AMACR.

2-Methyl-3-oxodecanoyl-CoA **25** was also a good inhibitor of AMACR. The α-proton of this compound is relatively acidic and **25** undergoes rapid non-enzymatic α-proton exchange with solvent *via* an enolate intermediate. It was therefore not possible to analyse the influence of 2-methyl group stereochemical configuration. The precursor **24** was a much poorer inhibitor than **25**, presumably due to reduced acidity of the α-proton. It is also possible that the additional steric bulk at carbon-3 contributes to the lower potency of **24** compared to **25**, as the 5-membered ring of **24** will be twisted out of plane relative to the aliphatic side-chain. However, AMACR is known to be able to accept substrates with diverse side-chain structures [6, 28, 36-38] and it is notable that ibuprofenoyl-CoA **5** and derivatives **6** - **9** (which have aromatic rings at the equivalent position) are potent inhibitors (Figure 1).

2*S*,3*R*-3-Hydroxy-2-methyldecanoyl-CoA **26** is also a relatively potent inhibitor. Incubation of **26** with active AMACR did not result in an elimination reaction, as judged by the lack of a peak at δ 1.75 ppm [10] from the 2-methyl group of the anticipated product **22** (Figure 2), probably because hydroxide is a relatively poor leaving group (water pK_a = ~7), compared to fluoride (HF pK_a = 3.2) [42]. Similarly, ^{1}H NMR analysis of the reaction products showed that **26** did not undergo α-proton exchange, and hence **26** is not a substrate of AMACR. It is notable that **22**, **24** and **26** are intermediates in the branched-chain acyl-CoA β-oxidation pathway. One may speculate that these compounds could provide some regulation of AMACR by negative feedback control, and hence control entry of *R*-2-methylacyl-CoA esters into the β-oxidation pathway. Whether or not this is physiologically significant will depend on rates of flux through the β-oxidation pathway and whether intermediates **22**, **24** and **26** are sequestered away from AMACR.

Acyl-CoAs which mimic the planar enolate intermediate are good inhibitors of AMACR (Figure 1). *E*-2-Methyldec-2-enoyl-CoA **22** and 2-methylenedecanoyl-CoA **23** bind strongly due to having a planar sp^2-hybridized α-carbon. This result is consistent with a previous study in which 2-methyleneacyl-CoAs were shown to be good competitive inhibitors [2, 30]. The enolate analogue *N*-dodecyl-*N*-methylcarbamoyl-CoA **4** is the best rationally designed inhibitor (IC_{50} = 0.4 nM) reported to date [29, 32]. Inhibition by **4** is *ca.* 2000× more potent than by **18** and **19** (Figure 1). This high potency of **4** appears to largely result from the mimicking of the enolate intermediate by the carbamoyl moiety. It is also notable that the determined IC_{50} value for **4** in this study (0.4 nM) is significantly lower than that previously determined by Carnell *et al*. (98 nM), when assayed against HEK-

derived human AMACR using ibuprofenoyl-CoA **5** as substrate [29]. It appears that the colorimetric assay consistently determines higher levels of compound potency than other assays (*e.g.* K_i = 60 nM [32] *vs*. 56 μM [28, 38] for an ibuprofenoyl-CoA epimeric mixture) The reasons for this discrepancy is not entirely clear, but higher apparent potency may be a consequence of using a substrate undergoing an irreversible reaction to measure activity (and hence avoiding the error introduced by the presence of the reverse reaction). Alternatively, this may be related to the extent of substrate or inhibitor micelle formation under the different assay conditions.

The determined IC_{50} value (0.4 nM) is around half the calculated 'active' enzyme concentration in the assay [32] (based on comparison of k_{cat}/K_m values for the *E. coli* and HEK cell derived enzymes [29]), and hence **4** could be behaving as a tight-binding inhibitor. Compound **4** behaves as a rapidly reversible competitive inhibitor of AMACR, with a Hill coefficient of ~0.7 [32]. These observations are consistent with the zone A inhibitor behaviour described by Straus and Goldstein [43, 44], *i.e.* the enzyme active site concentration is <0.1 × the apparent K_i value (0.65 nM [32]). This rapidly reversible inhibition is significantly different behaviour to that observed for similar compounds (*gem*-carbamoyl inhibitors and *N*-decyl-carbamoyl-CoA) with the highly homologous bacterial enzyme MCR, where time-dependent inactivation was observed [35]. The reasons for this difference in behaviour are not entirely obvious.

Influence of the side-chain lipophilicity on inhibitor potency

AMACR is able to catalyse the 'racemisation' of substrates with structurally diverse side-chains [1, 2]. The accommodation of these diverse structures is

thought to be a result of non-specific binding of the side-chain by hydrophobic interactions to a methionine-rich surface [8]. Consistent with this, the MCR *gem*- [31] and *gem*- carbamate [35] inhibitors show increased potency for compounds with more hydrophobic alkyl side-chains. Consequently, we were interested to investigate whether inhibitor potency was related to the lipophilicity of the inhibitor side-chain. A plot of determined IC_{50} values *vs.* calculated LogP values for the acyl-CoA inhibitor (Figure 3) showed that high LogP values tend to produce low IC_{50} values. Systematic trends were observed within those compounds containing aromatic side-chains (**5** - **11**), with potency positively correlating with lipophilicity. It is also notable that **10** and **11**, possessing a single phenyl group side-chain are significantly less potent than compounds containing more lipophilic side-chains. This consistent behaviour tends to suggest that side-chain lipophilicity is driving potency, with the 2-hydroxy group of **10** and **11** making a smaller contribution. Systematic trends were also observed for inhibitors possessing alkyl side-chains (**12** - **17**), showing that lipophilicity is also an important determinant of potency for this series.

The enolate analogue **4** has a potency increased by ~875-fold compared to that predicted based on LogP values (measured IC_{50} = ~0.4 nM *vs.* ~350 nM predicted for miLogP = 2.61) (Supplementary Information, Figure S1), showing the effectiveness of the carbamate moiety in promoting inhibition. Acyl-CoA esters do not comply with Lipinski guidelines and hence AMACR inhibitors are delivered as their acid pro-drugs [28, 30, 32, 34] which are converted to the acyl-CoA *in vivo*. Although **4** has very high potency, delivery as the pro-drug will be challenging because carbamates readily decarboxylate to the corresponding amine.

Conclusions

This is the first systematic SAR study of rationally designed AMACR inhibitors. The study illustrates that extremely diverse side-chain structures which can be accommodated. A minimal level of side-chain lipophilicity is required for efficient binding. For compounds with aromatic side-chains, a single aromatic ring results in modest inhibition whilst more than one aromatic ring or an aromatic ring with alkyl substituents results in much more potent inhibition. Similarly, a minimum of a 6-carbon alkyl chain appears to be required for reasonably efficient inhibitor binding, with increased potency resulting from addition of further $–CH_2–$ groups. Our results allow investigation of the contribution to potency made by the individual structural elements of these inhibitors.

AMACR has attracted much attention as both a novel drug target and cancer marker since its involvement in prostate cancer was reported [14, 19]. However, exploitation of this discovery has been extremely limited, largely due to the absence of a suitable assay with which to test inhibitor potency [33]. This study shows that our novel colorimetric assay [32] allows quick and accurate measurement of drug potency and detailed kinetic characterisation of inhibitors. The systematic investigation of novel inhibitor SAR and therapeutic development is now possible.

Materials and methods

Sources of materials

Chemicals were purchased from the Sigma-Aldrich Chemical Co. or Fisher Scientific Ltd., unless otherwise stated and were used without further purification. Reduced coenzyme A, *tri*-lithium salt was purchased from Calbiochem. Acyl-CoA

esters **4** [33]**, 5**-**9** [6], **10** and **11** [11] were synthesised as previously described. Acyl-CoA esters **12** – **17** were purchased from Larodan Lipids. Substrates **18-20** and product **22** were synthesised as described [7, 10]. Human recombinant AMACR 1A was expressed and purified and substrate **1** synthesised as previously described [32].

General experimental procedures

Thin layer chromatography was performed on Merck silica aluminium plates 60 (F254) and UV light, potassium permanganate or phosphomolybdic acid were used for visualisation. Column chromatography was performed using Fisher silica gel (particle size 35-70 micron). Purifications of acyl-CoA esters were performed by solid phase extraction using Oasis HLB 6cc (200 mg) extraction cartridges. Phosphate buffer was prepared from monobasic sodium phosphate and NaOH at the required proportions. Citric acid buffer was prepared from citric acid and NaOH at the required proportion for 0.8 M pH 4.0 buffer. The pH of aqueous solutions was measured using a Corning 240 pH meter and Corning general purpose combination electrode. The pH meter was calibrated using Fisher Chemicals standard buffer solutions (pH 4.0 - phthalate, 7.0 - phosphate, and 10.0 - borate) at either pH 7.0 and 10.0 or 7.0 and 4.0. Calibration and measurements were carried out at ambient room temperature. IR spectra were recorded on Perkin-Elmer RXI FTIR spectrometer instrument. NMR spectra were recorded on Bruker Avance III 400.04 MHz or 500.13 MHz spectrometers in D_2O, $(CD_3)_2SO$ or $CDCl_3$ and the solvent was used as an internal standard. Shifts are given in ppm and *J* values reported to $\pm$ 0.1 Hz. Multiplicities of NMR signals are described as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Stock

concentrations of acyl-CoA esters for assays were determined using ^{1}H NMR. Mass spectra were recorded by ESI TOF. High resolution mass spectra were recorded in ES mode. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. Syntheses were carried out at ambient temperature, unless otherwise specified. Solutions in organic solvents were dried over anhydrous $MgSO_4$ and the solvents were evaporated under reduced pressure. Aqueous solutions for biological experiments were prepared in Nanopure water of 18.2 MΩ.cm^{-1} quality and were pH-adjusted with aq. HCl or NaOH.

(4S)-4-Benzyl-3-[(2S,3R)-3-hydroxy-2-methyldecanoyl]-1,3-oxazolidin-2-one (28)

(*S*)-(+)-4-Benzyl-3-propanoyl-2-oxazolidinone **27** (3.0 g, 12.9 mmol) in CH_2Cl_2 (30.0 mL) was cooled to -78°C. Dibutylboron triflate (1.0 M in CH_2Cl_2, 13.0 mL, 12.9 mmol) and Pr^i_2NEt (2.3 mL, 12.9 mmol) were added and the mixture was stirred for 30 min before octanal (1.4 mL, 9.2 mmol) in CH_2Cl_2 (9.0 mL) was added dropwise. The mixture was stirred at -78°C for further 30 min and then at room temperature for 2 h. aq. Sodium phosphate buffer at pH 7.0 (100 mM, 100 mL) was added slowly to the reaction mixture. The organic layer was washed [aq. HCl (1.0 M), aq. $NaHCO_3$ (saturated), brine] and dried. Column chromatography (Petroleum ether / EtOAc 10:1 → 6:1) gave **28** (2.53 g, 76%) as a colourless oil. $[\alpha]_D^{21}$ +51.4 (*c* 0.74 in $CHCl_3$); IR ν_{max} 3517 (OH), 1780 (C=O), 1692 (C=O) cm^{-1}; ^{1}H NMR (500.13 MHz; $CDCl_3$) δ_H 7.26-7.07 (5 H, m, Ar-*H*), 4.65-4.55 (1 H, m, 4-*H*), 4.16-4.05 (2 H, m, 5-*H*), 3.89-3.80 (1 H, m, 3'-*H*), 3.68 (1 H, qd *J* = 7.0, 3.0 Hz, 2'-*H*), 3.14 (1 H, dd, *J* = 13.0, 3.0 Hz, C*H*HAr), 2.92 (1 H, s, O*H*), 2.70 (1 H,

dd, *J* = 13.0, 9.0 Hz, CH*H*Ar), 1.50-1.10 (15 H, m, 6 × C*H*$_2$ and C*H*$_3$CH), 0.79 (3 H, t, *J* = 6.5 Hz, 10'-H$_3$); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C177.23 (1'-*C*), 152.87 (2-*C*), 134.92 (Ar-*C*), 129.24 (Ar-*C*), 128.73 (Ar-*C*), 127.18 (Ar-*C*), 71.35 (3'-*C*), 65.96 (5-*C*), 54.91 (4-*C*), 42.02 (2'-*C*), 37.53 (*C*HHAr), 33.77 (*C*H$_2$), 31.62 (*C*H$_2$), 29.35 (*C*H$_2$), 29.05 (*C*H$_2$), 25.84 (*C*H$_2$), 22.45 (*C*H$_2$), 13.90 (*C*H$_3$CH), 10.31 (10'-*C*); ESI-MS *m/z* 384.2134 $[M + Na]^+$ ($C_{21}H_{31}NNaO_4$ requires 384.2151).

(4S)-4-Benzyl-3-[(2R,3S)-3-fluoro-2-methyldecanoyl]-1,3-oxazolidin-2-one (29)

(Diethylamino)sulfur trifluoride (0.5 mL, 3.9 mmol) in CH_2Cl_2 (10.0 mL) was added dropwise to **28** (1.4 g, 3.9 mmol) in CH_2Cl_2 (20.0 mL) at -78°C and the mixture was stirred for 2 h at this temperature. It was stirred for a further 2 h at room temperature, before being quenched with water (50 mL). The organic layer was washed (saturated aq. $NaHCO_3$, brine). Column chromatography (Petroleum ether / EtOAc 30:1 gave **29** (490 mg, 35%) as a colourless oil. $[\alpha]_D^{21}$ +49.2 (*c* 0.63 in $CHCl_3$); IR ν_{max} 1782 (C=O), 1700 (C=O) cm^{-1}; ^{1}H NMR (500.13 MHz; $CDCl_3$) δ_H 7.27-7.06 (5 H, m, Ar-*H*), 4.76-4.57 (2 H, m, 4-*H* and 3'-*H*), 4.15-3.96 (3 H, m, 5-*H* and 2'-*H*), 3.17 (1 H, dd, *J* = 13.5, 3.5 Hz, C*H*HAr), 2.72 (1 H, dd, *J* = 13.5, 9.5 Hz, CH*H*Ar), 1.70-1.14 (12 H, m, 6 × C*H*$_2$), 1.10 (3 H, d, *J* = 7.0 Hz, C*H*$_3$CH), 0.80 (3 H, t, *J* = 6.5 Hz, 10'-H$_3$); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ_C 174.37 (d, *J* = 2.8 Hz, 1'-*C*), 153.12 (2-*C*), 135.25 (Ar-*C*), 129.47 (Ar-*C*), 128.93 (Ar-*C*), 127.37 (Ar-*C*), 94.89 (d, *J* = 169.8 Hz, 3'-*C*), 66.19 (5-*C*), 55.38 (4-*C*), 42.03 (d, *J* = 20.9 Hz, 2'-*C*), 37.85 (*C*HHAr), 32.04 (d, *J* = 20.9 Hz, 4'-*C*H$_2$), 31.79 (*C*H$_2$), 29.37 (*C*H$_2$), 29.16 (*C*H$_2$), 24.57 (d, *J* = 2.8 Hz, 5'-*C*H$_2$), 22.64 (*C*H$_2$), 14.10 (10'-*C*) and

13.62 (d, *J* = 8.5 Hz, 2'-*C*H_3); δ_F (470 MHz, $CDCl_3$) -179.67; ESI-MS *m/z* 386.2126 $[M + Na]^+$ ($C_{21}H_{30}FNNaO_3$ requires 386.2107).

(2R,3S)-3-Fluoro-2-methyldecanoic acid (30)

H_2O_2 aq. [30% (w/w), 0.7 mL] and LiOH (62 mg, 2.6 mmol) were added to **29** (472 mg, 1.3 mmol) in THF (14 mL) at 0°C. The mixture was stirred at ambient temperature for 20 h before being quenched with sat. aq. sodium sulfite (14 mL). The THF was evaporated and the mixture was acidified with aq. HCl (1.0 M) to pH 1.0 and extracted with CH_2Cl_2 (100 mL). The organic phase was washed with water (75 mL) and brine (75 mL). The combined organic layers were dried and the solvent was evaporated. Column chromatography (petroleum ether / EtOAc 5:1) gave **30** (245 mg, 92%) as a white solid. mp 64-65°C; $[\alpha]_D^{21}$ -7.8 (*c* 0.51 in $CHCl_3$); IR ν_{max} 2925 (OH), 1693 (C=O) cm^{-1}; ^1H NMR (400.04 MHz; $CDCl_3$) δ_H 10.89 (1 H, br s, O*H*), 4.58-4.50 (1 H, m, 3-*H*), 2.58-2.52 (1 H, m, 2-*H*), 1.75-1.23 (12 H, m, 6 × CH_2), 1.20 (3 H, d, *J* = 7.2 Hz, CH_3CH), 0.88 (3 H, t, *J* = 6.8 Hz, 10-H_3); ^{13}C NMR (100.60 MHz, $CDCl_3$) δ_C 180.02 (d, *J* = 5.6 Hz, *C*=O), 94.28 (d, *J* = 172.2 Hz, 3-*C*), 44.37 (d, *J* = 22.0 Hz, 2-*C*), 31.74 (d, *J* = 21.1 Hz, 4-*C*H_2), 31.74 (*C*H_2), 29.30 (*C*H_2), 29.12 (*C*H_2), 24.82 (d, *J* = 3.0 Hz, 5-*C*H_2), 22.61 (*C*H_2), 14.05 (10-*C*) and 12.56 (d, *J* = 6.6 Hz, 2-*C*H_3); ^{19}F NMR (470 MHz, $CDCl_3$) δ_F -181.94; ESI-MS *m/z* 203.1449 $[M - H]^-$ ($C_{11}H_{20}FO_2$ requires 203.1447).

(2S,3S)-3-Fluoro-2-methyldecanoyl-CoA (21)

Carbonyldiimidazole (48 mg, 0.29 mmol) was added to (2*R*,3*S*)-3-fluoro-2-methyldecanoic acid **30** (30.0 mg, 0.15 mmol) in anhydrous CH_2Cl_2 (2 mL) and the mixture was stirred at ambient temperature for 1 h. CH_2Cl_2 (3 mL) was added

to the mixture, which was with water (5 × 2 mL) and brine (2 mL) and dried. The solvent was evaporated to obtain the crude acyl-imidazole intermediate. Aq. $NaHCO_3$ (1.0 mL, 0.10 M) and *tri*-lithium CoA-SH (17.0 mg, 0.02 mmol) was added to the crude intermediate in THF (1.0 mL) and the mixture was stirred at ambient temperature for 18 h. The THF was evaporated and the residue was acidified to *ca.* pH 3 by addition of aq. HCl (1.0 M HCl). The mixture was diluted with water (2.0 mL) and washed with EtOAc (3 × 3 mL). The crude aqueous solution was freeze-dried and purified with solid-phase extraction to give **21** (13.6 mg) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ_H 8.63 (1 H, s, adenosine C*H*), 8.38 (1 H, s, adenosine C*H*), 6.16 (1 H, d, *J* = 6.0 Hz, adenosine C*H*), 4.30-4.10 (2 H, m, adenosine C*H*2), 3.97 (1 H, s, adenosine C*H*), 3.85-3.72 (1 H, m, CoA(OC*H*H)), 3.58-3.47 (1 H, m, CoA(OCH*H*)), 3.39 (2 H, t, *J* = 6.5 Hz, CoA(C*H*2)), 3.29 (2 H, t, *J* = 6.0 Hz, CoA(CH_2)), 3.08-2.90 (3 H, m, CoA(SCH_2) and C*H*CH_3), 2.36 (2 H, t, *J* = 6.5 Hz, CoA(CH_2)), 1.68-1.44 (2 H, m, C*H*H and CH*H*), 1.39-1.12 (10 H, m, 5 × CH_2), 1.07 (3 H, d, *J* = 7.0 Hz, CHCH_3), 0.87 (3 H, s, CoA(CH_3)), 0.81-0.70 (6 H, m, CH_2CH_3 and CoA(C*H*3)); ^{19}F NMR (470 MHz) δ_F -181.11; ESI-MS *m/z* 475.6220 $[M - 2H]^{2-}$ ($C_{32}H_{53}FN_7O_{17}P_3S$ requires 475.6208).

2,2-Dimethyl-5-octyl-1,3-dioxane-4,6-dione (31)

4-Dimethylaminopyridine (1.296 g, 10.6 mmol), *N*,*N*'-dicyclohexylcarbodiimide (1.0 M in CH_2Cl_2, 11.1 mL, 11.1 mmol) and octanoic acid **32** (1.6 mL, 10 mmol, 1.0 eq.) were added to Meldrum's acid **33** (1.455 g, 10.1 mmol) in dry CH_2Cl_2 (100 mL). The mixture was stirred at ambient temperature for 40 h. The precipitate was removed by filtration and the filtrate was washed with aq. $KHSO_4$

(1.0 M, twice), water and brine, then dried. AcOH (6.0 mL) was added to the filtrate. To this solution, $NaBH_4$ (802 mg, 21.2 mmol) was added in portions during 1 h and the mixture was stirred for an additional 20 h. The evaporation residue was dissolved in Et_2O (100 mL) and washed with water (twice) and brine (100 mL). Drying and evaporation gave **31** (2.30 g, 89 %) as a white solid: mp. 64-65°C (lit. [45] mp. 65-67°C); ^1H NMR (400.04 MHz, $CDCl_3$): δ_H 3.49 (1 H, t, *J* = 4.9 Hz, dioxane 5-H), 2.14-2.04 (m, 2 H, octyl 1-H_2), 1.77 (3 H, s, 2-CH_3), 1.75 (3 H, s, 2-CH_3), 1.48-1.38 (2 H, m, octyl 3-H_2), 1.37-1.19 (10 H, m, octyl 4,5,6,7-H_8), 0.86 (3 H, t, *J* = 7.0 Hz, octyl 8-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ_C 165.6, 104.7, 46.1, 31.8, 29.5, 29.2, 29.1, 28.4, 26.9, 26.6, 26.5, 22.6, 14.1; IR (KBr disc) n_{max} 1752 (C=O) cm^{-1}; ESI-MS *m/z* 279.1577 $[M + Na]^+$ $C_{14}H_{24}NaO_4$ requires 279.1572; 257.1732 $[M + H]^+$ ($C_{14}H_{25}O_4$ requires 257.1753).

Methyl 2-methylenedecanoate (34)

Compound **31** (2.10 g, 8.19 mmol) was dissolved in anhydrous MeOH (26 mL). Eschenmoser's salt (3.79 g, 20.5 mmol, 2.5 eq.) was added and the mixture was heated at reflux for 40 h. The solvent was evaporated. The residue, in Et_2O (100 mL), was washed with aq. $KHSO_4$ (1.0 M), water and brine and was dried. Evaporation and column chromatography (petroleum ether / EtOAc 10:1) gave **34** (1.10 g, 74 %) as a colourless oil (lit.[46] oil): IR (neat) n_{max} 1725 (C=O) cm^{-1}; ^1H NMR (400.04 MHz, $CDCl_3$): δ_H 6.12-6.10 (1 H, m, =C*H*H), 5.52-5.49 (1 H, m, =CH*H*), 3.73 (3 H, s, OCH_3), 2.31-2.23 (2 H, m, 3-H_2), 1.49-1.37 (2 H, m, 4-H_2), 1.34-1.19 (10 H, m, 5,6,7,8,9-H_{10}), 0.86 (3 H, t, *J* = 7.1 Hz, 10-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ_C 167.8, 140.8, 124.4, 51.7 (two carbons), 31.8, 29.3, 29.2, 29.2, 28.3, 22.6, 14.0.

2-Methylenedecanoic acid (35)

Methyl ester **34** (870 mg, 4.39 mmol) was stirred at 50 °C for 2 h with aq. NaOH (1.0 M, 15 mL, 15 mmol) in EtOH (57 mL), then cooled to ambient temperature and acidified to pH *ca.* 3. The volatile solvents were evaporated. The residue, in Et_2O (50 mL), was washed with water (twice) and brine and was dried. Evaporation and column chromatography (petroleum ether / EtOAc 3:1) gave **35** (650 mg, 80 %) as a colourless oil (lit. [47] oil): IR (neat) n_{max} 1696 (C=O) cm^{-1};^{1}H NMR (400.04 MHz, $CDCl_3$): δ_H 12.05 (1 H, br s), 6.34-6.23 (1 H, m, =C*H*H), 5.70-5.59 (1 H, m, =CH*H*), 2.32-2.25 (2 H, m, 3-H_2), 1.54-1.40 (2 H, m, 4-H_2), 1.36-1.19 (10 H, m, 5,6,7,8,9-H_{10}), 0.87 (3 H, t, *J* = 7.0 Hz, 10-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ_C 173.0, 140.2, 126.9, 31.8, 31.4, 29.3, 29.2, 29.2, 28.3, 22.6, 14.1; ESI-MS *m/z* 207.1349 $[M + Na]^+$ $C_{11}H_{20}NaO_2$ requires 207.1361); 183.1396 $[M]^-$ ($C_{11}H_{19}O_2$ requires 183.1385).

2-Methylenedecanoyl-CoA (23)

Ethyl chloroformate (17 µL, 19 mg, 0.18 mmol) was added to **35** (33 mg, 0.18 mmol) and NEt_3 (25 µL, 18 mg, 0.18 mmol) in anhydrous THF (2.0 mL) and the mixture was stirred at ambient temperature for 1 h. CoA-SH tri-lithium salt [(28 mg, 0.04 mmol) in aq. $KHCO_3$ (2.5%), 2.0 mL] was added and the mixture was stirred at ambient temperature for 16 h. The mixture was acidified to pH *ca.* 3 with aq. HCl (1.0 M) and the THF was evaporated. The solution was washed with EtOAc (5 × 3 mL) and the crude product was purified by SPE to give **23** (7.0 mg) as a colourless solid: ^{1}H NMR (500.13 MHz, D_2O): δ_H 8.47 (1 H, s), 8.16 (1 H, s), 6.07 (1 H, d, *J* = 7.0 Hz), 5.96 (1 H, s), 5.55 (1 H, s), 4.19-4.11 (1 H, m), 3.75 (1 H, dd, *J* = 9.8, 5.1 Hz), 3.46 (1 H, dd, *J* = 9.8, 4.8 Hz), 3.36 (2 H, t, *J* = 6.5 Hz),

3.31-3.25 (2 H, m), 2.95 (2 H, t, *J* = 6.2 Hz), 2.33 (2 H, t, *J* = 6.6 Hz), 2.16 (2 H, t, *J* = 7.3 Hz), 1.30-1.22 (2 H, m), 1.18-1.06 (10 H, m), 0.79 (3 H, s), 0.75 (3 H, t, *J* = 6.8 Hz), 0.65 (3 H, s); ESI-MS *m/z* 465.6152 $[M - 2\ H]^{2-}$ ($C_{32}H_{52}N_7O_{17}P_3S$ requires 465.6177).

Methyl 2-(2-heptyl-1,3-dioxolan-2-yl)propanoate (36)

Ethane-1,2-diol (5.27 g, 84.8 mmol) was added to **37** (606 mg, 2.83 mmol) in dry CH_2Cl_2 (26 mL). Me_3SiCl (1.84 g, 2.15 mL, 17.0 mmol) was added and the mixture was stirred at ambient temperature for 3 d. Further ethane-1,2-diol (5.27 g) and Me_3SiCl (1.84 g) were added and the mixture was stirred for a further 3 d. Water (25 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with water (5 × 70 mL) and brine (70 mL) and were dried. Evaporation and column chromatography (petroleum ether / EtOAc 10:1) gave **36** (600 mg, 82%) as a colourless oil: IR (neat) ν_{max} 1740 (C=O) cm^{-1}; ^1H NMR (400.04 MHz, $CDCl_3$) δ_H 4.04-3.90 (4 H, m, OCH_2CH_2O), 3.67 (3 H, s, OCH_3), 2.83 (1 H, q, *J* = 7.2 Hz, C*H*CH_3), 1.81-1.62 (2 H, m, heptyl 1-H_2), 1.42-1.20 (10 H, m, heptyl 2,3,4,5,6-H_{10}), 1.18 (3 H, d, *J* = 7.2 Hz, propanoate 3-H_3), 0.86 (3 H, t, *J* = 7.1 Hz, heptyl 7-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ_C 173.9, 111.3, 65.5, 65.4, 51.7, 46.7, 35.0, 31.7, 29.7, 29.2, 22.8, 22.6, 14.0, 12.5; ESI-MS *m/z* 281.1761 $[M + Na]^+$ ($C_{14}H_{26}NaO_4$ requires 281.1729), 259.1883 $[M + H]^+$ ($C_{14}H_{27}O_4$ requires 259.1909).

2-(2-Heptyl-1,3-dioxolan-2-yl)propanoic acid (38)

Aq. NaOH (1.0 M, 6.7 mL, 6.7 mmol) was stirred with **36** (345 mg, 1.34 mmol) in MeOH (30 mL) at ambient temperature for 2 h, then at 65°C for 2 h. The mixture

was cooled to ambient temperature and citric acid buffer (0.8 M, pH 4.0, 15 mL) was added. The mixture was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layers were washed with water and brine and dried. Evaporation and column chromatography (petroleum ether / EtOAc 2:1) gave **38** (130 mg, 40 %) as a colourless oil. IR (neat) n_{max} 1709 (C=O) cm^{-1}; ^1H NMR (400.04 MHz, $CDCl_3$): δ_H 10.78 (1 H, br s, OH), 4.10-3.94 (4 H, m, OCH_2CH_2O), 2.83 (1 H, q, *J* = 7.2 Hz, C*H*CH_3), 1.80-1.67 (2 H, m heptyl 1-H_2), 1.43-1.20 (10 H, m, heptyl 2,3,4,5,6-H_{10}), 1.23 (3 H, d, *J* = 7.2 Hz, CHC*H*$_3$), 0.87 (3 H, t, *J* = 7.1 Hz, heptyl 7-H_3); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ_C 176.9, 111.4, 65.4, 65.4, 46.6, 34.7, 31.7, 29.6, 29.2, 22.8, 22.6, 14.0, 12.2; ESI-MS *m/z* 267.1549 $[M + Na]^+$ ($C_{13}H_{24}NaO_4$ requires 267.1572), 245.1729 $[M + H]^+$ ($C_{13}H_{25}O_4$ requires 245.1753).

2-(2-Heptyl-1,3-dioxolan-2-yl)propanoyl-CoA (24)

Using the same method as for **21**, **24** was prepared from **38** (31 mg, 0.13 mmol, 1.0 eq.) by sequential treatment with *N*,*N*'-carbonyldiimidazole (41 mg, 0.25 mmol, 2.0 eq.) and CoA-SH tri-lithium salt (29 mg, 0.04 mmol, 0.3 eq.) to give **24** (10 mg) as a colourless solid: ^1H NMR (500.13 MHz, D_2O) δ_H 8.57 (1 H, s), 8.30 (1 H, s), 6.13 (1 H, d, *J* = 6.1 Hz), 4.21-4.14 (1 H, m), 3.99-3.88 (4 H, m), 3.78 (1 H, dd, *J* = 9.6, 4.5 Hz), 3.50 (1 H, dd, *J* = 9.7, 4.5 Hz), 3.39 (2 H, t, *J* = 6.6 Hz), 3.32-3.22 (2 H, m), 3.12 (1 H, 2 x q, *J* = 7.0 Hz; both epimers), 2.93 (2 H, t, *J* = 6.2 Hz), 2.36 (2 H, t, *J* = 6.6 Hz), 1.66-1.53 (2 H, m), 1.26-1.11 (8 H, m), 1.08 (3 H, d, *J* = 7.0 Hz, $CHCH_3$), 0.85 (3 H, s), 0.76 (3 H, t, *J* = 7.1 Hz, CH_2CH_3), 0.72 (3 H, s); ESI-MS *m/z* 506.6219 $[M + Na]^{2+}$ ($C_{34}H_{55}N_7NaO_{19}P_3S$ requires 506.6192), 495.6301 $[M + H]^{2+}$ ($C_{34}H_{56}N_7O_{19}P_3S$ requires 495.6282).

2R,S-2-Methyl-3-oxodecanoyl-CoA (25)

Compound **24** (7 mg, 7.1 μmol) was dissolved in water (0.8 mL) and acetone (1.0 mL). Aq. HCl (1.0 M, 0.2 mL) was added and the reaction mixture was stirred overnight. The acetone was evaporated and the residue freeze-dried to give **25** (6 mg) as a colourless solid: ^{1}H NMR (500.13 MHz, D_2O): δ_H 8.49 (1 H, s), 8.33 (1 H, s), 6.10 (1 H, d, *J* = 5.8 Hz), 4.20-4.10 (2 H, m), 3.99 (1 H, q, *J* = 7.0 Hz), 3.76 (1 H, dd, *J* = 9.7, 5.0 Hz), 3.38-3.28 (2 H, m), 3.22 (2 H, t, *J* = 6.3 Hz), 2.97-2.88 (2 H, m), 2.57-2.44 (2 H, m), 2.30 (2 H, t, *J* = 6.6 Hz), 1.41-1.32 (2 H, m), 1.16 (3 H, d, *J* = 7.0 Hz), 1.12-1.04 (8 H, m), 0.80 (3 H, s), 0.69 (3 H, s), 0.68 (3 H, t, *J* = 7.0 Hz); ESI-MS *m/z* 473.6174 $[M - 2H]^{2-}$ ($C_{32}H_{52}N_7O_{18}P_3S$ requires 473.6151).

2S,3R-3-Hydroxy-2-methyldecanoyl-CoA (26)

2*S*,3*R*-3-Hydroxy-2-methyldecanoic acid **39** was synthesised [48] from the Evan's auxiliary protected acid **28** by hydrolysis with NaOH and H_2O_2. Following the procedure used for **21**, 2*S*,3*R*-3-hydroxy-2-methyldecanoyl-CoA **26** was prepared from 2*S*,3*R*-3-hydroxy-2-methyldecanoic acid **39** (40 mg, 0.20 mmol, 1.0 eq.), CDI (64 mg, 0.40 mmol, 2.0 eq.) and CoA-SH *tri*-lithium salt (78 mg, 0.10 mmol, 0.5 eq.) to give **26** (12 mg) as a colourless solid: ^{1}H NMR (500.13 MHz, D_2O) δ_H 8.48 (1 H, s), 8.20 (1 H, s), 6.09 (1 H, d, *J* = 6.5 Hz), 4.22-4.10 (1 H, m), 3.81-3.70 (1 H, m), 3.48 (1 H, dd, *J* = 9.8, 4.8 Hz), 3.37 (2 H, t, *J* = 6.6 Hz), 3.25 (2 H, t, *J* = 6.3 Hz), 2.97-2.87 (2 H, m), 2.74-2.68 (1 H, m), 2.34 (2 H, t, *J* = 6.5 Hz), 1.39-1.29 (2 H, m), 1.21-1.09 (10 H, m), 1.06 (3 H, d, *J* = 6.9 Hz, $CHCH_3$), 0.81 (3 H, s), 0.74 (3 H, t, *J* = 7.1 Hz, CH_2CH_3), 0.68 (3 H, s); ESI-MS *m/z* $[M - 2H]^{2-}$ 474.6217 ($C_{32}H_{54}N_7O_{18}P_3S$ requires 474.6229).

Evaluation of inhibition of AMACR by test compounds

Colorimetric assays were performed as previously described [32]. Dose response curves were used to determine IC_{50} values for inhibitors. Enzyme (4 x stock, 150 µL) and inhibitor at the appropriate concentration (4 x stock, 150 µL) were incubated together in 96 well plates at ambient room temperature for 10 min. The sample was divided into three repeats of 100 µL before addition of substrate (2 x stock, 3 x 100 µL; final concentration of 40 µM in the assay) and monitored at 354 nm. Each 200 µL assay contained *ca.* 8 µg of total AMACR protein (0.85 µM, assuming a molecular weight of 47,146.8 Da. with one active site per monomer [7]). Final concentrations of inhibitor in the assay were 100, 33.3, 11.1, 3.7, 1.23, 0.411, 0.137 and 0.045 µM unless otherwise stated. Positive controls contained enzyme and substrate **1** only and negative controls buffer and substrate. Rates in ΔAbsorbance.min^{-1} were determined using Excel and converted to nmol.min.$^{-1}$mg^{-1} using the 2,4-dinitrophenoxide **2** extinction coefficient (15,300 M^{-1} cm^{-1}) [32] with the path-length (0.588 cm) determined by the plate-reader. IC_{50} values were determined using reaction rate, with the data fitted to a 4-parameter logistic using SigmaPlot 13 using Log_{10} inhibitor concentration (in µM). In some cases 2-3% (v/v) DMSO was included in assays; no significant change in enzyme activity was observed with DMSO concentrations of up to 8% (v/v) [32]. Half-volume 96 well plates were used for some inhibitors; identical IC_{50} values were obtained for standard inhibitors using both types of microtitre plate. IC_{50} values [32] for *N*-dodecyl-*N*-methylcarbamoyl-CoA **4** and ibuprofenoyl-CoA **5** were determined contemporaneously with the inhibitors described in this study, with the same batch of enzyme.

Computational analysis of potency of inhibition

Lipophilicity of acyl-CoA esters was assessed by calculation of miLogP values using the molecular properties calculator (http://www.molinspiration.com/cgi-bin/properties). miLogP values were calculated using molecular smiles obtained from Chemdraw Professional 15. IC_{50} values (in nM) were plotted against the obtained LogP value using SigmaPlot 13.

Acknowledgements

This work was funded by Prostate Cancer UK (S10-03 and PG14-009), a University of Bath Overseas Research Studentship and a Biochemical Society Summer Vacation studentship (2015). The authors are members of the Cancer Research @ Bath (CR@B) network.

Disclosure statement

MJ, GLL, MDT, TJW and MDL are named inventors on patent applications on the use and application of the colorimetric assay. The other authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Appendix A Supplementary Information

Supplementary Information contains dose-response curves and other biological evaluation of inhibitors and spectroscopic characterisation data for compounds. Supplementary Information can be found in the online version at:

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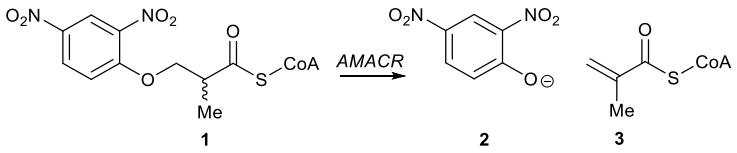
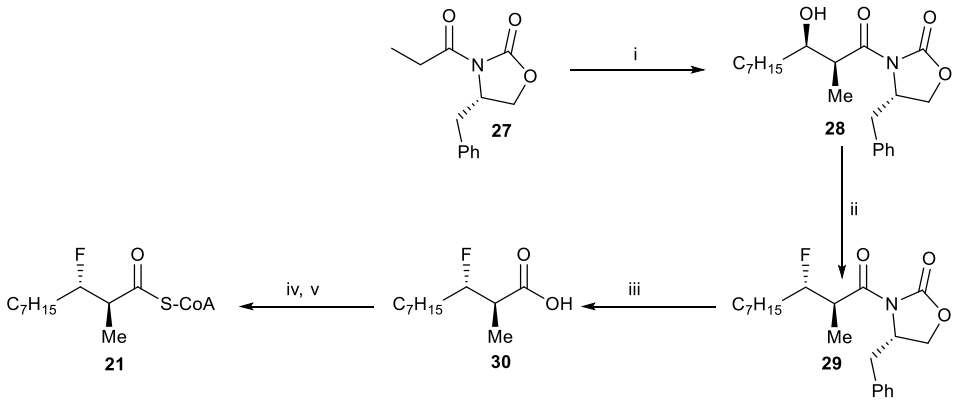
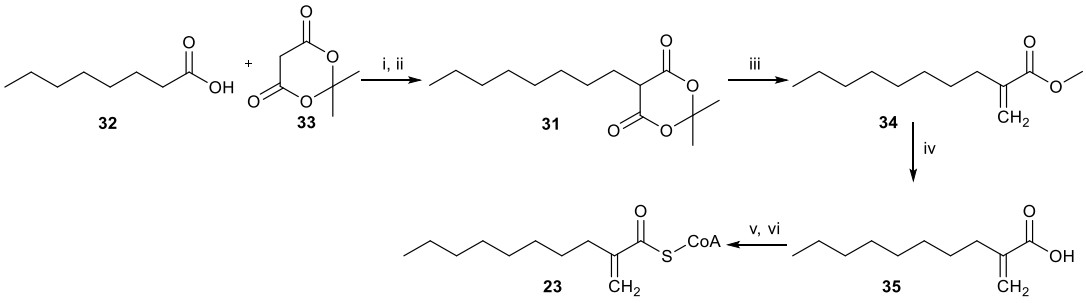
Schemes and figures



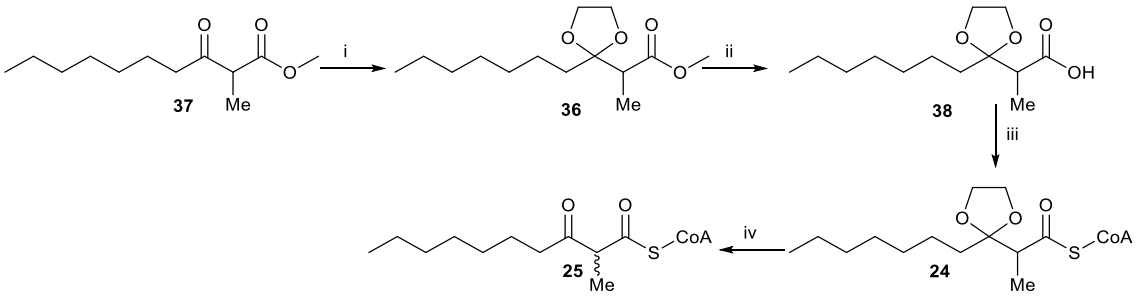
Scheme 1.



Scheme 2.



Scheme 3.



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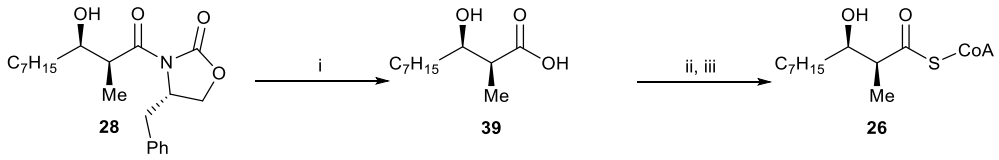

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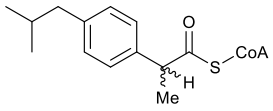

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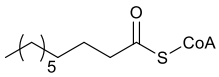

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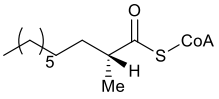

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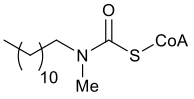

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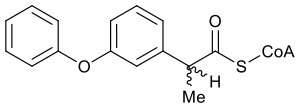

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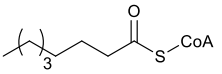

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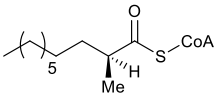

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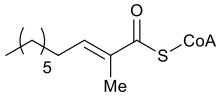

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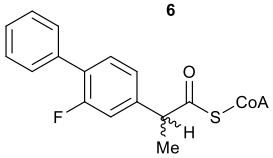

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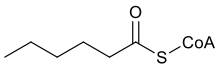

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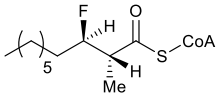

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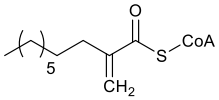

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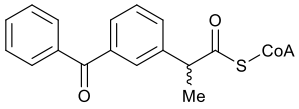

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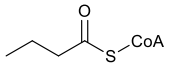

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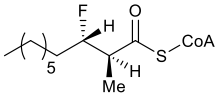

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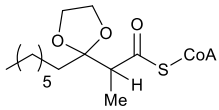

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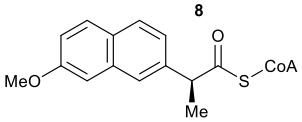

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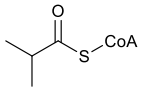

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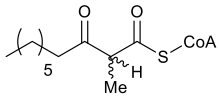

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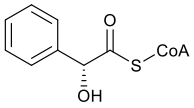

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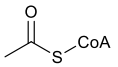

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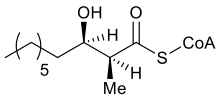

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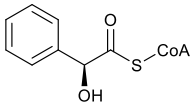

11

Compound	*IC_{50} (nM)*	*miLogP**	*Previously tested as substrate?*	*Previously tested as Inhibitor?*
4	~0.4	-2.61	No	Yes [29, 32]
5	540	-4.18	Yes [6, 29, 49]	Yes [28, 32, 37, 38]
6	400	-3.77	Yes [6, 11]	Not tested
7	590	-3.62	Yes [6]	Not tested
8	560	-4.07	Yes [6]	Not tested
9	750	-4.25	Yes [6]	Not tested
10	3.8×10^3	-5.46	Not a substrate [11]	Not tested
11	2.3×10^3	-5.46	Not a substrate [11]	Not tested
12	3.1×10^3	-3.63	Yes [36]	Not tested
13	9.6×10^3	-4.50	Yes [36]	Yes – No inhibition [37, 38]
14	1.6×10^4	-4.98	Yes [36]	Not tested
15	$>1.0 \times 10^5$	-5.30	Poor substrate [36]	Yes – No inhibition [37, 38]
16	$>1.0 \times 10^5$	-5.38	Not a substrate [36]	Yes – No inhibition [37, 38]
17	$>1.0 \times 10^5$	-5.55	Not tested	Yes – No inhibition [37, 38]
18	930	-3.39	Yes [7]	Not tested
19	1170	-3.39	Yes [7, 36]	Not tested
20	200	-3.61	Yes [10]	Not tested†
21	300	-3.61	Not tested	Not tested†
22	180	-3.34	Not a substrate [10]	Yes – No inhibition [37, 38]
23	600	-3.56	Not tested	Not tested‡
24	4.1×10^3	-4.02	Not tested	Not tested
25	360	-4.55	Not tested	Not testedǁ
26	560	-4.43	Not tested	Not tested

Figure 1.

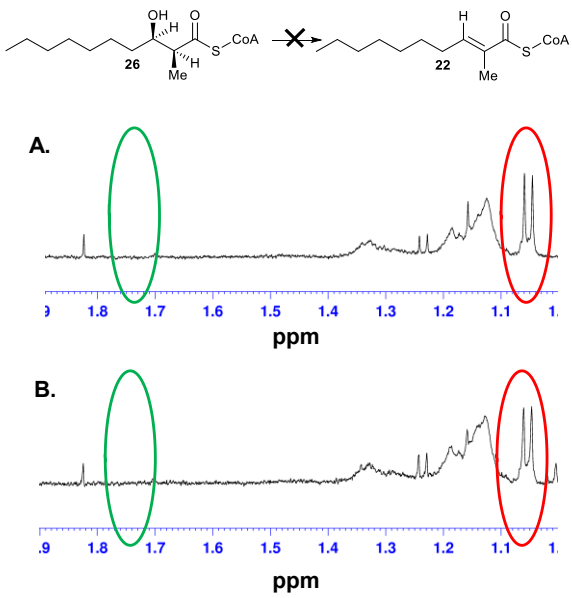
OH
H
O
S
CoA
26
Me
H
H
O
S
CoA
22
Me
A.
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
ppm
B.
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
ppm

Figure 2.

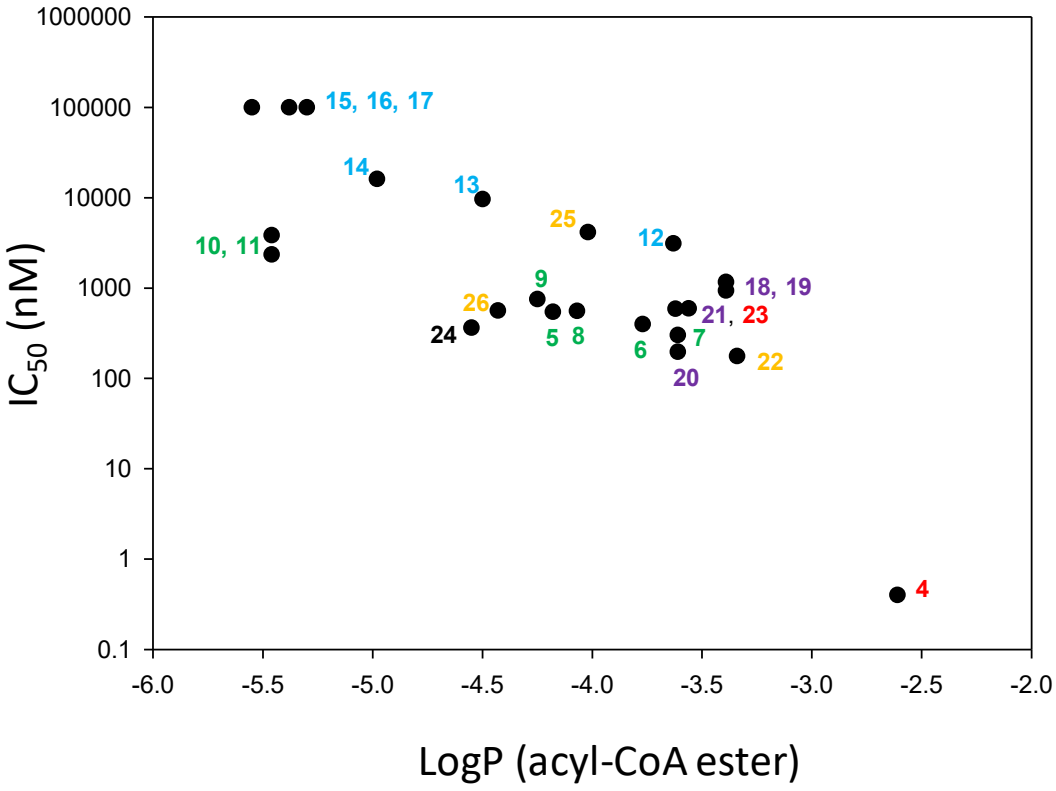
1000000
100000
10000
1000
100
10
1
0.1
IC50 (nM)
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
LogP (acyl-CoA ester)
15, 16, 17
14
13
25
12
10, 11
9
18, 19
26
21, 23
24
5
8
6
7
22
20
4

Figure 3.

Legends

Scheme 1. The colorimetric assay for AMACR 1A [32] showing elimination of 2,4-dinitrophenolate **2**.

Scheme 2. Synthesis of (2*S*,3*S*)-3-fluoro-2-methyldecanoyl-CoA **21.** Reagents and conditions: i. Bu_2BOTf, Pr^i_2NEt, octanal, CH_2Cl_2, -78°C, 76%; ii. DAST, CH_2Cl_2, -78°C, 35%; iii. LiOH, H_2O_2, H_2O/THF, 0°C, 92%; iv. CDI, CH_2Cl_2, rt; v. CoA-SH Li^+_3, 0.1 M $NaHCO_{3\ aq.}$ /THF (1:1).

Scheme 3. Synthesis of 2-methylenedecanoyl-CoA **23**. Reagents and conditions: i, DCC, DMAP, CH_2Cl_2; ii, $NaBH_4$, AcOH, 89% over two steps; iii, $Me_2N^+=CH_2$ I^-, MeOH, 74%; iv, NaOH, EtOH, 80%; v, NEt_3, EtOCOCl, THF; vi, aq. $KHCO_3$ (2.5% w/v), CoA-SH Li^+_3, THF.

Scheme 4. Synthesis of 2-methyl-3-oxodecanoyl-CoA **25**. Reagents and conditions: i, ethane-1,2-diol, TMSCl, CH_2Cl_2, 82%; ii, aq. NaOH / MeOH, 40%; iii, CDI, CH_2Cl_2; v, CoA-SH Li^+_3, 0.1 M $NaHCO_{3\ aq.}$/THF (1:1); iv, aq. HCl, acetone.

Scheme 5: Synthesis of (2*S*,3*R*)-3-hydroxy-2-methyldecanoyl-CoA **26.** Reagents and conditions: i. NaOH, H_2O_2, rt, quant.; ii. CDI, CH_2Cl_2, rt; iii. CoA-SH Li^+_3, 0.1 M $NaHCO_{3\ aq.}$ /THF (1:1).

Figure 1. Structures for AMACR inhibitors, as measured by the colorimetric assay [32]. *Calculated miLogP values are for the acyl-CoA ester (http://www.molinspiration.com/cgi-bin/properties). †(2*R*,3*R*)- and (2*S*,3*S*)-3-Fluoro-2-methylhexadecanoyl-CoA were previously shown to be AMACR inhibitors [28]. ‡Several side-chain analogues of **23** reported as inhibitors [30]. ‖Binding of the 2-methylacetoacetyl-CoA enolate to MCR observed by X-ray crystallography [9].

Figure 2. Incubation of 2*S*,3*R*-3-hydroxy-2-methyldecanoyl-CoA **26** with AMACR in buffer and $^{2}H_2O$. A. Heat-inactivated enzyme; B. live enzyme. Red circles highlight doublet for substrate 2-methyl group, showing no exchange of the α-proton had occurred (conversion to a single peak occurs on exchange to α-^{2}H upon 'racemisation' [7, 11]). Green circles denote expected position of 2-methyl singlet for the expected unsaturated product **22**, showing that no elimination reaction has occurred.

Figure 3. Correlation of inhibitor potency with lipophilicity (miLogP value; http://www.molinspiration.com/cgi-bin/properties). Compound numbers refer to structures shown in Figure 1. Compounds with green numbers are those with aromatic side-chains (**5** – **11**); Compounds with blue numbers are straight-chain acyl-CoA esters and iso-butanoyl-CoA (**12** – **17**); Compounds with purple numbers are 2-methyldecanoyl-CoA and 3-fluoro-2-methyldecanoyl-CoAs (**18** - **21**); Compounds with orange numbers are intermediates in the β-oxidation pathway which occurs subsequent to AMACR activity (**22**, **25** and **26**);

Compounds with red numbers are inhibitors or analogues of known inhibitors (**4** and **23**); Compound **24** is a synthetic intermediate to **25**.